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NON-PROVISIONAL
UTILITY PATENT APPLICATION
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Washington, DC 20231

Attorney Docket No. 4070-174US (K400417US)
First Named Inventor: Horst Peschel
Express Mail Label No. EL474257472US
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Transmitted herewith for filing is the non-provisional utility patent application entitled:

**Brain Tissue Derived from Neuronal Precursor Cells, it's use as Transplant and a Method
of Preparation thereof**

Which is:

an Original; or
a Continuation, Divisional, or Continuation-in-part (CIP)
of prior Application No. /, filed .

This non-provisional patent application is based on Provisional Patent Application
No. , filed .

Enclosed are:

- Specification (including Abstract) and claims: 21 pages.
- Newly unexecuted Declaration (original).
- Copy of Declaration from prior application.
- Separate Power of Attorney (including 37 CFR 3.73(b) statement, if applicable).
- 1 sheets of drawings (formal) plus one copy.
- Microfiche computer program (Appendix).
- Nucleotide and/or Amino Acid Sequence Submission, including:
 - Computer readable copy Paper Copy Verified Statement.
- Under PTO-1595 cover sheet, an assignment of the invention.
- Certified copy of Application No., filed , is filed:
 - herewith or in prior application .
- Verified Statement Claiming Small Entity Status under 37 CFR 1.9 and 1.27.
 - was filed in the prior non-provisional application, and such
status is still proper and desired (37 CFR 1.28(a));
 - is enclosed herewith; is no longer desired.
- Preliminary Amendment.
- Information Disclosure Statement, PTO-1449, and cited references.
- Other:

The filing fee has been calculated as shown below:

			SMALL ENTITY		LARGE ENTITY	
CLAIMS	NO. FILED	NO. EXTRA	BASIC FEE: \$345		BASIC FEE: \$690	
Total	25-20 =	5	X9	\$	OR	X18
Independent	3- 3=	0	X39	\$	OR	X78
Multiple Dependent Claims Present:			\$130	\$	OR	\$260
			TOTAL	\$	OR	TOTAL

The Commissioner is hereby authorized to charge payment of the following fees or credit any overpayment to Deposit Account No. 50-1017. One additional copy of this sheet is enclosed.

- The above calculated filing fee 0 - (**Billing No. 204070.0174**).
- Any additional fees required under 37 C.F.R. § 1.16.
- Any additional fees required under 37 C.F.R. §1.17.
- If the filing of any paper during the prosecution of this application requires an extension of time in order for the paper to be timely filed, applicant(s) hereby petition(s) for the appropriate extension of time pursuant to 37 C.F.R. §1.136(a).

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PATENT
Attorney Docket
No. 4070-174US
(K400417US)

TITLE OF THE INVENTION

Brain Tissue Derived From Neuronal Precursor Cells, It's Use As Transplant And
A Method Of Preparation Thereof

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BACKGROUND OF THE INVENTION

This invention relates to brain tissue, which is developed from immature neuronal precursor cells as a source for tissue transplantation in neurological and neurosurgical disorders and the method of generating such tissue.

Restorative treatment strategies have been have been exploited in 10 respect to many neurological and neurosurgical disorders. The underlying idea is to replace dead or non-functional tissue by appropriate cell suspensions. There is a chance to treat, e. g. Parkinson's disease with Implants consisting of dopaminergic neurons, Alzheimer's disease with cholinergic neurons, Huntington's disease with striatal GABAergic neurons and multiple system atrophy with dopaminergic and 15 GABAergic neurons.

Unfortunately, these treatment options are limited by the lack of appropriate tissue, which is derived from human embryos.

In addition, there is debate about the need for immunosuppressive 20 therapy following transplantation. Most current protocols involve immunosuppression causing significant side effects.

Tissue derived from human embryos also needs to be transplanted into patients within a few days severely limiting microbiological and genetical testing.

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SUMMARY OF THE INVENTION

The invention is based on the problem to develop a source of brain tissue, which can be used for transplantation therapy or from which such transplants can easily be derived. This tissue must not bear any of the above mentioned 30 disadvantages. In addition, this invention includes the problem to present a method for manufacturing such tissue.

This problem is achieved by the independent claims described below.

This invention is based on the concept that neuronal progenitor (precursor) cells can be isolated and expanded in vitro. The proliferation of said neuronal progenitor cells can be modulated using appropriate substances, e. g. 5 proteins, in a way that these cells become determined to differentiate completely or predominantly into a specific cell type (e. g. dopaminergic neuron) after transplantation or, in general, after making contact with an appropriate substrate such as other cells or a supporting material. Using this treatment, one can generate 10 tissue cultures that almost substantially contain immediate precursors of specific neurons. These cultures do not include cells that give rise to immunogenic glial cells in large enough quantities to induce any detectable immune response. Thus, the inventor can generate appropriate well-characterized tissue for transplantation with virtually unlimited supply.

15 This invention allows to generate tissue that substantially contains dopaminergic and/or cholinergic and/or GABAergic and/or serotonergic neurons alone or any combination thereof. The percentage of such specific neurons in the tissue samples should be greater than 90 %, preferably greater 95 %. Thus, the tissue does not contain other cells, e. g. glial cells, which would be physiologically 20 relevant.

Neuronal progenitor cells from which the issue for transplantation is derived can be isolated from embryonic or adult brain or spinal cord preparations. If an adult donor is used, neuronal progenitor cells are preferably isolated from subventricular or hippocampal brain regions.

25 Neuronal progenitor cells are abundant in embryonic brain tissue. Thus, brain regions may be selected that normally contain the neurons of interest. Neuronal progenitor cells that differentiate into dopaminergic neurons may best be isolated from midbrain tissue. This invention, however, allows to generate different 30 determined progenitor cells from the same pluripotent progenitor cell pool, which may also be derived from umbilical cord blood.

Most efficiently, neuronal progenitor cells are prepared from human embryonic brain tissue, 3- 25 weeks of gestation, preferably 5 – 11 weeks of gestation.

Isolation and culturing of neuronal Progenitor cells from rodent brain has been reported (Daadi und Weiss, J. Neurosci 1999; Magrassi et al., Development 1998; 54:107-115; Ptak et al., Cell Transplant 1995; 4:299-310; Liepelt et al., Brain Res Dev Brain Res 1990; 51:267-278).

5 Neuronal progenitor cells were successfully isolated from various parts of the brain. In addition, neuronal progenitor cells could also be isolated from human embryonic brain tissue (Buc-Caron, Neurobiol Dis 1995; 2:37-47; Svendsen CN et al., Exp Neurol 1997; 148:135-146; Sah et al., Nat Biotechnol 1997; 15:574-580; Chalmers-Redman et al., Neuroscience 1997; 76:1121-1128. The technique of 10 preparation of brain tissue and isolation of neuronal progenitor cells has been adapted from these protocols.

15 Tissue that can be used for transplantation of patients is prepared according to the invention which includes the expansion of direct or indirectly harvested progenitor cells, partial differentiation in vitro and a selection of cells. The resulting tissue cultures differentiate into specific cell types preferably without addition application of compounds or genetic engineering.

20 A population of determined neuronal progenitor cells that have been selected and partially differentiated maintains the ability to perform mitosis allowing for performing subsequent proliferation step.

25 Partial differentiation and selection may be performed repeatedly with possible variation among individual treatments.

30 This invention finally allows to modulate immature pluripotent neuronal progenitor cells to become highly determined progenitor cells that will predominantly or only differentiate into a specific cell type after transplantation or in vitro differentiation.

35 Expansion of neuronal progenitor cells may include a variation of atmospheric oxygen content, priming, transient or non-transient expression of foreign genes, treatment with exogenous compounds especially under reduced oxygen partial pressure, or a combination of these. These individual treatments will be explained in detail below.

40 The selection of determined progenitor cells includes generation of clonal cell lines, which may include a variation, especially a reduction, of atmospheric oxygen.

The procedure may include selective expansion of freshly isolated progenitor cells. Proliferation of selected cells may be promoted using a modulation of atmospheric oxygen content, or by application of appropriate mitogens or by priming with exogenous compounds that stimulate differentiation, if desired each under reduced oxygen content of the atmosphere. Before or after priming cells may be subcloned, if desired under reduced oxygen partial pressure. In addition, transient expression of foreign genes may be used to promote further determination of individual clonal cell lines. The effect induced by the reduction of atmospheric oxygen content may be simulated or enhanced using conditions that exert similar effects on cell metabolism (e. g. inhibitors of mitochondrial energy production such as rotenone, MPP+ or malonate). If desired a further expansion or further partial differentiation by the methods mentioned above can be conducted.

Expansion of determined progenitor cells preferably originates from a single cell.

The success of selection of determined cell lines, which is the characterization after complete *in vivo* (after transplantation) or *in vitro* differentiation is performed using cytometric, biochemical, molecular biology, immunohistochemical and/or electrophysiological methods (see below).

Expansion using a modulation of atmospheric oxygen content:

The rate of proliferation of neuronal progenitor cells can be increased using a reduction of oxygen and/or an increase of nitrogen concentrations in the incubator. In addition, these modulations of culturing conditions promote the proliferation of specific neurons (e. g. dopaminergic neurons). At present, human embryonic midbrain derived progenitor cells may only be expanded using such conditions (Fig. 1). For example, the oxygen content may be lowered from 20 % (room air) to 10 %, better 5 % or preferably 1 %, especially with a corresponding increase in nitrogen content. However, addition of other gaseous compounds is possible. The reduction of the oxygen content is performed when cells are supplemented with mitogens, which is the expansion state. As mentioned above similar effects may be obtained using inhibitors of mitochondrial respiration.

The exogenous mitogens (detailed description below) maybe used in concentrations varying from 4000 to 0.01 ng/ml, better 500 to 1 /ml, preferably 100 to 2 ng/ml. Concentrations outside these ranges are not excluded.

Partial differentiation using priming:

Priming includes intermittent treatment of (monoclonal) neuronal progenitor cells with one or more compounds that promote differentiation in specific neurons. These compounds include e. g. growth factors, cytokines, neurotransmitters. In addition, conditioned media may be employed. These media may be derived from primary cultures containing striatal, glial or other brain cells or used to cultivate these neurons. The media contain aminoacid compounds being secreted from these cells. Preferably cells of the target region of the neurons of choice are used. To generate tissue for transplantation these media may be serum-free. These compounds are removed after a period (preferably a few hours) that allows dedifferentiation into progenitor cells that maintain their capability to perform mitosis. Said primed progenitor cells respond to a subsequent treatment with such factors more rapidly. Said primed progenitor cells may be subcloned and/or expanded. Priming may be repeated several times using identical or alternative combinations and/or concentrations of differentiation promoting compounds.

Priming may be performed with a variety of substances (exogenous factors). One may use combinations of cytokines and growth factors, cytokines and neurotransmitters, cytokines and hormones, cytokines and gangliosides, cytokines and conditioned media, growth factors and neurotransmitters, growth factors and hormones, growth factors and gangliosides, growth factors and conditioned media, neurotransmitters and hormones, neurotransmitters and gangliosides, neurotransmitters and conditioned media, etc.

Any of the above mentioned combinations may again be combined. Growth factors comprise one or more of the epidermal growth factor (EGF) family, preferably EGF1, EGF2, or EGF3 including α and β subgroups, transforming growth factor (TGF) α and β , LIN-3, fibroblast growth factor (FGF) 1 and 2, nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophines (NT) 3,4,5 and 6, insulin like growth factor (IGF) 1 and 2, glial cell line-derived neurotrophic factor (GDNF), Neurturin (NTN), Persephin (PSP), vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF) including all members of individual families and proteins with similar mode of action.

Cytokines may include one or a combination of leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), the family of interleukines (IL1-16), tumor necrosis factor (TNF), especially TNF α , interferones (IFN), especially IFN- α , macrophage inhibitory or stimulating factor, especially macrophage migration inhibitory factor (MIF), mitochondrial import stimulation factor (MSF) and retinoic acid.

Treatment with neurotransmitters may include one or a combination of dopamine, acetylcholine, GABA, glutamate, glycine, taurin, proline, noradrenaline, serotonin and various neuropeptides such as substance P and enkephaline.

In addition one or combinations of various hormones, especially steroid hormones or thyroid hormone, gangliosides, and their derivatives may be used.

Priming of neuronal progenitor cells in order to determine these to differentiate into dopaminergic neurons preferably includes treatment with GDNF, LIF, IL1, IL11 and/or thyroid hormone.

These exogenous compounds may be administered in concentrations ranging from 25,000 bis 0.005 ng/ml, better 1000 bis 0.1 ng/ml, preferably 100 bis 1 ng/ml expansion media. Concentrations outside these ranges are not excluded.

Especially, IL-1 concentrations of 0.005 to 10 ng/ml, preferably 0.01 to 2 ng/ml, especially between 0.05 to 0.25 ng/ml could be used. IL-11 and LIF could be applied in concentrations of 0.01 to 100 ng/ml, preferably 0.1 to 20 ng/ml, most preferably between 0.5 to 2.5 ng/ml. GDNF could be applied in concentrations from 1 to 25,000 ng/ml, preferably 1-10 to 5,000 ng/ml, most preferably between 1-100 to 2,500 ng/ml.

These concentrations of exogenous factors may also be used when combinations of any of these are employed. However, the actual concentrations are not limited to the above mentioned ranges and may vary depending on the combination.

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Partial differentiation using transfection:

The generation of highly determined neuronal progenitor cells may also include genetic engineering, especially in combination with priming. Using transfection with genes that are known to be crucial for the development of specific

cell types, a high degree of determination may be achieved. A transient transfection is preferred since integration of plasmid DNA into the chromosomal DNA of such cells is not warranted to avoid administration of foreign genes into the host brain.

5 Determination to differentiate into dopaminergic neurons may be promoted via expression of members of the steroid or thyroid hormone receptors, tyrosine hydroxylase, NURR1 and/or NURR77. In addition, genes encoding for the vesicular monoamine transporter (VMAT2) or the dopamine transporter may be used. In general, all genes that play a role in the development of such neurons may be employed.

10 To select for neuronal progenitor cells that are determined to differentiate into cholinergic neurons, genes of the nicotinic acetylcholine receptor family, NGF receptors or cholinesterase can be used.

15 Progenitors determined to differentiate into GABAergic neurons may be generated via transient transfection with dopamine receptor, glutamat receptor, γ amino butyric acid transporters enkephaline and/or substance P genes.

All of the above mentioned cDNA's are known and available. Transfection may be performed using standard procedures resulting in transient or stable expression of these genes.

20 Generation of monoclonal cell lines:

25 To select for determined cell lines, one may generate monoclonal cell lines. Using appropriate protocols it is possible to generate cell lines that are derived from a single cell(monoclonal cell line). Subcloning has been proven useful to minimize the heterogeneity of various cell suspensions. Using rat embryonic midbrain derived monoclonal cell lines individual clones could be identified of which 98 % of the cells expressed proteins which are specific or dopaminergic neurons (tyrosine hydroxylase) following in vitro differentiation.

Subcloning may be performed using dilutions of single cell suspensions or may be aided using fluorescence-activated cell sorting (FACS) after labeling of vital cells or via enriching these cell suspensions using a magnetic column after labeling the cells with superparamagnetic beads or micromanipulation.

Subcloning with suspensions of single cells may be performed using gravity extraction of non-dissociated cells and dilution of the remaining single cells to a concentration calculated to contain only one cell per volume that is needed for

plating. Cells are plated in expansion media allowing for proliferation of monoclonal cell lines. Said monoclonal cell lines will be treated and characterized as described above.

5 Micromanipulation may be performed to increase the yield of monoclonal determined cell lines. Viable cells will be labeled with a fluorescent marker that is specific for the respective cell population. Using fluorescent microscopy single fluorescent cells can be identified and selected with appropriate tools such as a glass capillary. These cells may then give rise to monoclonal cell lines which have a much higher potential to be determined to differentiate into the cell type of choice. Preferably mitogenic substances (see above) are added to the expansion media. Differentiation and characterization is conducted as described below. Neuronal progenitor cells that express the tyrosine hydroxylase gene may be labeled using expression of a fluorescent protein (e. g. EGFP) which is controlled by the promoter of the tyrosine hydroxylase or dopamine transporter gene. Similar 10 approaches may be used to identify neuronal progenitor cells that ready express specific proteins using the choline-acetyl-transferase promoter (cholinergic marker), glutamyl transferase (GABAergic marker) or other respective promoter elements. In addition, viable cells may also be identified using fluorescent antibodies to specific membrane localized proteins (dopamine transporter, nicotinic acetylcholine 15 receptors, especially α , β subunits (especially α 7 subunits) GABA transporter, etc.).

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Similar labeling techniques may also be employed to isolate specific neuronal progenitor cells using FACS. Said labeled cells may be separated from unlabeled cells using standard FACS protocols (for review: Orfao und Ruiz-Arguelles, Clin Biochem 1996;29:5-9). These isolated cells may be expanded using 25 polyclonal cell lines or via subcloning of monoclonal cell lines after dilution of single cell suspensions.

Magnetic isolation of determined neuronal progenitor cells may be 30 performed using labeling of viable cells with superparamagnetic beads. These beads are commercially available (Basic microbeads - dextran coated with free amines, 50nm, Miltenyi Biotech; Amino/Carboxy beads, 110-140 nm, Immunicon Corp.; Streptavidin/Biotin coated, Miltenyi Biotech or Immunicon Corp.).

Ligands for specific proteins (e. g. dopamine D2 receptor, dopamine transporter, nicotinic acetylcholine receptors, GABA transporter, serotonin

transporter, etc.) may be fused to the surface of these beads. Suspensions of individual cells may be incubated with these loaded beads. After binding of the magnetic beads to individual cells, these cells can be isolated via contact with a magnetic column. When the magnet in the column is turned off, cells that express the desired proteins can be eluted and used to generate polyclonal or monoclonal cell lines. Neuronal progenitor cells that express dopaminergic neuron specific proteins such as dopamine receptors or dopamine transporter may be identified using spiperone- or benzamide derivates as ligands for dopamine D2 receptors or cocaine derivatives as ligand for the dopamine transporter. For labeling of GABAergic cells, ligands for the GABA transporter may be used. Cholinergic cells may be recognized using ligands for acetylcholine receptors.

All of the above mentioned procedures are used to isolate (subclone) cells which are highly determined to differentiate into a given cell type but maintain their ability to divide. These procedures include priming with exogenous factors that stimulate differentiation, expression of foreign genes and/or changes in atmospheric oxygen and nitrogen concentrations.

Expansion after subcloning, micromanipulation, magnetic isolation and/or FACS is always performed using identical or similar expansion media as described above.

All of the above mentioned procedures may be combined or repeated.

Isolation and expansion of neuronal progenitor cells:

1. Fetal and Adult Progenitor Cell Cultures (Expansion):

Embryonic brain tissue from 5 to 12 weeks after gestation may be acquired under compliance with German Ärztekammer guidelines, germane government guidelines, and the local ethics committee and appropriate consent forms were used. Samples may be collected and the forebrain and ventral mesencephalon including the subependymal region may be dissected. To confirm the origin of midbrain samples, a small amount of tissue should be processed further for primary culture and stained for tyrosine hydroxylase (TH). The tissue samples may be serially incubated with serine protease such as trypsin (50 – 500 mg/ml) for 30 min at 23°C and DNase (20 - 60 µg/ml) for 2 - 30 min at 37°C, mechanically triturated to a quasi-single cell suspension and plated into un-coated 25 cm²-flasks (0.05 – 10

$\times 10^6$ cells per flask) in 5 ml expansion media, supplemented with efficient concentrations of mitogens (EGF, 10 - 100 ng/ml and/or FGF2, 5 to 100 ng/ml or others). Cultures may be placed in a humidified incubator at 37°C and 5% CO₂, 95 % air or at lowered O₂ conditions using a O₂-sensitive electrode system. Growth factors will be supplemented every other to every second day and cultures will be passed every 10 to 20 days. Cells may be labeled with BrdU with 10 μ M BrdU (Sigma) 2,4 and 7 days after plating for 24 h. The expansion media may contain mitogens and 10 – 60 % F12 or 30 – 60 % Dulbecco's Modified Eagle's Medium (DMEM; without glucose or with various glucose concentrations), efficient concentrations of an antibiotic (50 to 250 units/ml penicilline and 50 to 250 μ g/ml streptomycine). IN addition, the expansion media may contain one or combinations of the following compounds: transferring, diamines, especially putrescine,, sodiumselenit, gestagens, especially progesterone or similar compounds and insulin. Commercially available mixtures of supplements such as B27 (Gibco) may be used instead or in addition.

2. Expansion in altered atmospheric conditions: Normal room air contains about 20 % oxygen and less than 1 % CO₂. Tissue culturing is usually performed in air that contains the same amount of oxygen (20 %) and 1-10, preferably about 5 % CO₂. The rate of proliferation of various neuronal progenitor cells depends on this air composition. As indicated above human midbrain derived progenitor cells and cells that differentiate into dopaminergic neurons are specifically promoted using reduced oxygen condition. Neuronal progenitor cells may, therefore, be incubated in adequate systems that allow tight and continuous control not only of CO₂ but also oxygen content and indirectly nitrogen content using a O₂-sensitive electrode system. Oxygen concentrations may vary between less than 1 % and 30 %. To promote proliferation of progenitor cells determined to differentiate into dopaminergic neurons 1% - 5 % oxygen are preferably employed.

3. Transfection of Cells with Genes: One may transfet cells during proliferation to prepare these cells for cell sorting. Plasmid DNA may be added in concentrations of 0.1 – 5 μ g / ml, preferably 0.5 – 1.0 μ g of plasmid DNA per ml content of tissue culture flask and appropriate amounts of commercially available transfection reagents, e. g. 3 μ l per μ g DNA of TransFast (Promega) solution (prepared according to the instructions of the manufacturer). This solution may be incubated at 37°C and then added to the tissue culture flasks. To identify

differentiated neurons one may incubate DNA and transfection solutions in complete differentiation media 1 hour at 37°C. Cells may be harvested, washed and resuspended in the differentiation media containing plasmid DNA and lipofectin for another hour at 37°C before plating on precoated tissue culture dishes.

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Characterization of neuronal progenitor cells before and after differentiation:

1. Measurement of Proliferation by $[^3\text{H}]$ Thymidin Incorporation and Protein Determination: $[^3\text{H}]$ Thymidin incorporation and protein determination may be carried out according to standard procedures reported in the literature.

10

2. Measurement of Proliferation and Viability by Flow

Cytometry: Since progenitor cells are sensitive to mechanical stress and grow in neurospheres, which may be difficult to homogenized to single cell suspension, cell count and viability check can not be performed using standard procedures. One may use electronic cell analysis (e. g. CASY® TTC system). This system is based on flow cytometry and allows for measuring cell count, whole cell volume, cell volume, and various other cell parameters.

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3. Differentiation of Progenitor Cells: Cells may be

differentiated in vitro by plating them onto poly-L-lysine-coated cover slips or 48 well-plates in neurobasal media (Gibco). Media may be supplemented with FCS, cytokines and/or striatal-conditioned media. The following cytokines will be used: Interleukin 1b (IL-1b), IL-11, leukemia inhibitory factor (LIF), and glial cell line-derived factor (GDNF) or other exogenous factors (described above in respect to priming). The cells are allowed to differentiate for 7 to 10 days at 37°C in a humidified atmosphere before fixation and immunostaining.

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4. Toxicological Assays: *MTT assay*: After incubation of the

cultures with the substance of interest, 30 μl of MTT reagent (0.5 mg/ml MTT in PBS containing 10 mM HEPES) may be added to each well and incubated in at 37°C for 2 h. The medium is aspirated from each well and the culture plate dried at 37°C for 1 h. The resulting formazan dye can be extracted with 100 μl acid-isopropanol and the absorbency measured spectrophotometrically using computer-operated immuno reader at a wavelength of 570 nm with reference at 630 nm. Wells without cells will be used as blanks and are subtracted as background from each sample. *Trypan blue exclusion method*: The trypan blue assay will be carried out according to standard procedures.

5. Immunocytochemistry: Cultures may be fixed using 3.7% paraformaldehyde and washed with PBS. After blocking with normal serum, primary antibody may be added and incubated over night at 4°C. The following day, primary antibody may be removed and biotinylated secondary antibody added for 1 h followed by visualization via the ABC system coupled to nickel/DAB/H₂O₂ reaction or fluorescence-conjugated antibody. All cultures may be incubated with secondary antibody without primary antibody to ensure the specificity of the reaction. All plates may be assessed for the distinct staining by an individual blinded to treatment history. For visualization of dopamine cells anti-tyrosine hydroxylase and anti-dopamine transporter antibodies may be used, for GABAergic cells antibodies against anti-GAD65 & 67, for cholinergic cells antibodies against ChAT, for glial cells anti-GFAP antibodies, for neurons anti-MAP2 and anti-β-tubulin III, for oligodendrocytes anti-O4 antibodies may be used. For anti-BrdU staining to demonstrate proliferation of the cells the method according to the manufacturer (RPN-20 kit; Amersham) may be used. For double staining (in particular anti-BrdU and TH, anti-tubuline and TH or GAD) to demonstrate specific neuronal and glial phenotypes from progenitor cell origin, immunfluorescence stained cultures may be assessed using the fluorescence microscope equipped with visual analysis system (Axiovert 135; Zeiss).

6. Transmitter High-Affinity Uptake Studies: Functional integrity of DA and GABA neurons may be evaluated by measuring the uptake of their respective tritiated neurotransmitter. After preincubation for 10 min in incubation buffer containing 100 μM pargyline, 1 mM ascorbate, and 2 mM β-alanine (and for determination of nonspecific uptake: 3 μM GBR12909 and 1 mM 2,4-diamino-*n*-butyric acid; DABA), 50 nM [³H]DA, [³H]choline or [³H]GABA may be added for 15 min at 37°C. Uptake may be stopped by washing the dishes with cold PBS and the remaining radioactivity in the cell lysate may be measured using liquid scintillation counting. Specific uptake may be defined as the difference between the uptake measured in the absence (total) and the uptake measured in the presence of GBR12909 and DABA (nonspecific).

7. Determination of Dopamine, Acetylcholine and GABA by HPLC: For determination of dopamine, GABA and acetylcholine a HPLC-based method may be used. Determination of acetylcholine and GABA may be performed using standard procedures.

CLAIMS

I claim:

1. Isolated brain derived tissue not containing any physiologically active amounts of immunocompetent glial cells.
2. Said tissue described in claim 1, almost substantially consisting of dopaminergic neurons, cholinergic neurons, GABAergic neurons, and/or serotonergic neurons or differentiate into these neurons.
3. Said tissue described in claim 1, substantially consisting of dopaminergic neurons or cells that can differentiate into dopaminergic neurons.
4. Said tissue described in claim 1, substantially consisting of cholinergic neurons or cells that can differentiate into cholinergic neurons.
5. Said tissue described in claim 1, substantially consisting of GABAergic striatal neurons or cells that can differentiate into GABAergic striatal neurons.
6. Said tissue described in claim 1, substantially consisting of serotonergic neurons or cells that can differentiate into serotonergic neurons.
7. Said tissue described in claim 1, further characterized that the tissue is derived from mammals, especially humans.
8. Said tissue described in claim 7, further characterized that this is derived from developing immature (progenitor) cells.
9. Tissue that is useful to restore neuronal deficits after transplantation generated according to claim 1.
10. Monoclonal cell line derived from mammalian, especially human, progenitor cells characterized by exclusive or predominant differentiation into neurons when exposed to differentiation promoting factors.

11. Method to generate an expandable tissue culture derived from progenitor cells comprising the following individual steps:

dissection of mammalian brain tissue
isolation of progenitor cells
proliferation of progenitor cells
partial differentiation of progenitor cells
if necessary repetition of at least one of the steps isolation, proliferation and partial differentiation

12. Method according to claim 11, further characterized by selecting individual cells using subcloning

13. Said method described in claim 12, using one or multiple of the following procedures in suitable sequences:

subcloning using final dilution
subcloning using micromanipulation
subcloning using fluorescence activated cell sorting
subcloning using labeling and isolation with super-paramagnetic beads.

14. Said method described in claim 11, further characterized by the fact that the selection and partial differentiation of progenitor cells may be performed in conditions with reduced or increased oxygen/ nitrogen content or conditions that simulate reduced or increased oxygen/nitrogen content.

15. Said method described in claim 12, further characterized by the fact that oxygen content is less than 10 %, preferably less than 5 %.

16. Said method described in claim 12, further characterized by the fact that the conditions induced by reduced oxygen content may be simulated by application of compounds such as inhibitors of mitochondrial energy production.

17. Said method described in claim 11, further characterized by the fact that the progenitor cells are partially differentiated using expression of foreign genes or priming.

18. Said method described in claim 11, further characterized by the fact that the progenitor cells are partially differentiated by priming using treatment with appropriate cytokines, growth factors, hormones, neurotransmitters, transcription factors and/or gangliosides for periods of time that induce expression of tissue specific genes but do not preclude proliferation.

19. Said method described in claim 18, further characterized by the fact that priming is induced by one or a combination of members of the following growth factor families: EGF, FGF, GDNF, TGF α and β , LIN-3-protein, NGF, BDNF, NT, PDNF, IGF and/or VEGF, including respective subunits.

20. Said method described in claim 18, further characterized by the fact that priming is induced by one or a combination of the following cytokines: LIF, CNTF, interleukines (IL1-16), interferones, MIF, MSF, retinoic acid.

21. Said method described in claim 18, further characterized by the fact that priming is induced by one or a combination of the following neurotransmitters: dopamine, acetylcholine, GABA, glutamate, glycine, taurine, proline, noradrenaline, serotonin, substance P, enkephaline.

22. Said method described in claim 18, further characterized by the fact that priming is induced in monoclonal progenitor cell lines.

23. Said method described in claim 11, further characterized by the fact that proliferation of progenitor cells is achieved under hypoxic conditions or addition of exogenous factors.

24. Tissue culture generated according to claim 18.

25. Tissue capable of restoring neuronal deficits following transplantation generated according to claim 24.

ABSTRACT OF THE DISCLOSURE

This invention relates to neuronal tissue which is suitable to restore neuronal deficits following transplantation. To reduce immunological side effects and to increase the microbiological and genetic safety of the tissue we propose tissue that does not contain glial cells and is maintained in vitro for prolonged periods. The method to generate expandable determined neuronal progenitor cell cultures includes the following procedures: dissection of appropriate mammalian brain regions, isolation of progenitor cells, expansion of progenitor cells, selection and expansion of individual cells and priming.

ABSTRACT OF THE DISCLOSURE

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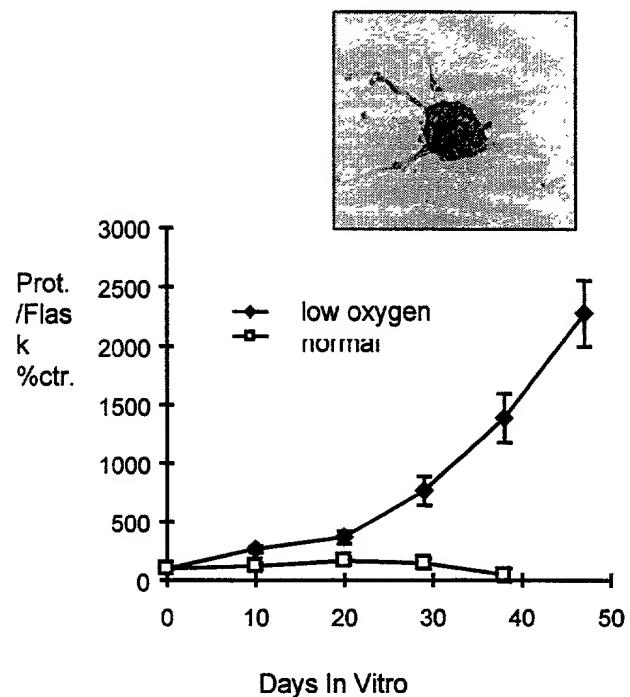


Figure 1: Cell proliferation of human midbrain derived progenitor cells under normal and atmospheric conditions with normal or reduced oxygen assessed by total protein content per flask. The top panel shows a representative phase contrast microphotograph of a cell cluster („neurosphere“) of progenitor cells after 20 days in culture.

DECLARATION AND POWER OF ATTORNEY
(Original Application)

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

Brain Tissue Derived from Neuronal Precursor Cells, it's use as Transplant and a Method of Preparation thereof

the specification of which is attached hereto and/or was filed on June 19, 2000 application No.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to herein.

I acknowledge the duty to disclose information which is material to patentability in accordance with Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d), of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

FOREIGN PRIORITY APPLICATION(S)

			Priority Claimed
199 28 210.2 (Number)	Germany (Country)	19 June 1999 (Day/month/year filed)	[X] Yes [] No

I hereby claim the benefit under Title 35, United States Code §119(e) of any United States provisional patent application(s) listed below and have also identified below any

United States provisional patent application(s) having a filing date before that of the application on which priority is claimed:

PROVISIONAL PRIORITY PATENT APPLICATION(S)

Priority Claimed

[] Yes [] No

(Application No.)

(Filing Date)

And I hereby appoint the registered attorneys and agents associated with **AKIN, GUMP, STRAUSS, HAUER & FELD, L.L.P.**, Customer No. 000570, as my attorneys or agents with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

Address all correspondence to **Customer No. 000570, namely, AKIN, GUMP, STRAUSS, HAUER & FELD, L.L.P.**, One Commerce Square, 2005 Market Street, Suite 2200, Philadelphia, Pennsylvania 19103. Please direct all communications and telephone calls to Gary D. Colby at 215-965-1285.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Full name of sole inventor, if any Horst Peschel

Inventor's Signature _____

Date _____

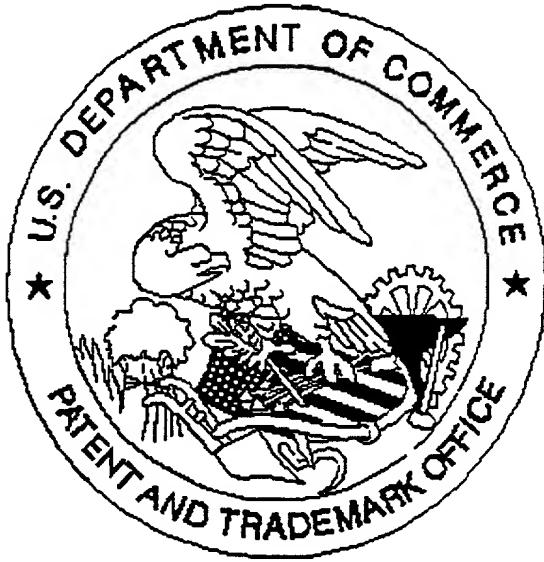
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